Video Article

Single-cell Gene Expression Using Multiplex RT-qPCR to Characterize Heterogeneity of Rare Lymphoid Populations

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Abstract

Gene expression heterogeneity is an interesting feature to investigate in lymphoid populations. Gene expression in these cells varies during cell activation, stress, or stimulation. Single-cell multiplex gene expression enables the simultaneous assessment of tens of genes[1,2,3]. At the single-cell level, multiplex gene expression determines population heterogeneity[4,5]. It allows for the distinction of population heterogeneity by determining both the probable mix of diverse precursor stages among mature cells and also the diversity of cell responses to stimuli.

Innate lymphoid cells (ILC) have been recently described as a population of innate effectors of the immune response[6,7]. In this protocol, cell heterogeneity of the ILC hepatic compartment is investigated during homeostasis.

Currently, the most widely used technique to assess gene expression is RT-qPCR. This method measures gene expression only one gene at a time. Additionally, this method cannot estimate heterogeneity of gene expression, since multiple cells are needed for one test. This leads to the measurement of the average gene expression of the population. When assessing large numbers of genes, RT-qPCR becomes a time-, reagent-, and sample-consuming method. Hence, the trade-offs limit the number of genes or cell populations that can be evaluated, increasing the risk of missing the global picture.

This manuscript describes how single-cell multiplex RT-qPCR can be used to overcome these limitations. This technique has benefited from recent microfluidics technological advances[1,2]. Reactions occurring in multiplex RT-qPCR chips do not exceed the nanoliter-level. Hence, single-cell gene expression, as well as simultaneous multiple gene expression, can be performed in a reagent-, sample-, and cost-effective manner. It is possible to test cell gene signature heterogeneity at the clonal level between cell subsets within a population at different developmental stages or under different conditions[4,5]. Working on rare populations with large numbers of conditions at the single-cell level is no longer a restriction.

Video Link

The video component of this article can be found at https://www.jove.com/video/54858/

Introduction

Over the past few years, innate lymphoid cells (ILCs) have been increasingly investigated. Despite their lack of antigen-specific receptors, they belong to the lymphoid lineage and represent important sentinels for tissue homeostasis and inflammation. ILCs are currently divided into three groups based on their expression of specific transcription factor combinations and on their ability to produce cytokines[6,7].

ILCs contribute to numerous homeostatic and pathophysiological situations in diverse organs via specific cytokine production[8,9]. To be able to understand the role of these cells, it is important to determine the various ILC subpopulations per organ and to identify their developmental relationships. In addition, phenomena of plasticity between the different subsets have been related. By studying the heterogeneity of the cells present in one organ, it is possible to delimit their stage of maturation and to distinguish their specific functions.

To illustrate the technique of single-cell multiplex RT-qPCR, hepatic ILCs were chosen, with a particular emphasis on their heterogeneity within the same ILC group (type 1 ILC)[10]. First, through the use of flow cytometry, three distinct ILC populations were characterized in the liver. Group 1 ILC represents around 80% of the innate effectors, while the two other populations are rare hepatic ILC populations (less than 5% of the innate effectors). Those populations were sorted using widely expressed cell-surface markers of ILC populations. As a result, sorted ILC populations in the liver look broadly similar one to another.

Single-cell multiplex RT-qPCR has emerged as one of the best techniques to promptly investigate the heterogeneity of these populations[11]. Two main characteristics are determined by taking advantage of the single-cell multiplex RT-qPCR technique. First, by looking at the clonal level, it is possible to recover cell-specific gene expression for comparison between cells that apparently display similar developmental stages. Then, by looking at a pre-selected combination of gene expression, we will determine new gene signatures based on simultaneous gene expression

patterns at one time point. These aspects permit the collection of a wide variety of expression data for a large number of cells, even on rare populations, since the technique is performed at the clonal level. Thereby, ILC heterogeneity in the liver can be adequately assessed.

Next, by sorting all cells with a global ILC phenotype, a wide overview of the multiple-gene expression of the liver ILC populations is obtained, even though they represent extremely rare populations. A microfluidic-based chip allows experimentation with even a small amount of cell material. As a consequence, the gene expression profiles of rare cell populations can be obtained. Using online gene signature analysis software, cell population clusters and potential cell relationships can be investigated. Consequently, functional tests can be performed to validate the clustering data at the *in vivo* level.

Tens of gene expressions could be assessed concomitantly on hundreds or more single cells on the same chip[3,11,12]. Design of the assay is the longest and most important part of the experiment. The determination of the genes relevant to the hypothesis to be tested is paramount to obtain relevant results. Secondly, internal control (such as known surface markers used for sorting) and specific controls are needed. This is crucial to test the primer amplification specificity, the efficiency of the amplification, and the absence of primer competition. Therefore, working with single-cell multiplex RT-qPCR is a timesaving technique, as multiple-gene expression of a cell is assessed at the same time.

Using the same chip and mix of reagents for all cells limits the possible errors of manipulation and allows for reproducibility between samples. Altogether, the different aspects of single-cell multiplex RT-qPCR allows for the production of highly reliable results at the clonal level, with a great level of sensitivity for a wide variety of samples and genes. The obtained results offer powerful and robust data for biostatistical tests.

This can be achieved due to the microfluidic aspect of the method, which allows for work on very small amounts of material and leads to exhaustive results. Finally, using online software, it is possible to compare the desired populations.

Protocol

All animal experiments were approved of by the Pasteur Institute Safety Committee in accordance with the French Agriculture Ministry and the EU guidelines.

1. Prepare a 96-well Single-cell Sorting Plate

1. Prepare pre-amplification mix in a 1.5 ml tube by adding 5.0 µl of specific retro-transcription buffer, 1.3 µl of low ethylenediaminetetraacetic acid (EDTA) TE buffer (10 mM TE solution, pH 8, and 0.1 mM EDTA solution; 0.2 µM filtered), and 0.2 µl of Taq DNA polymerase per well (**Figure 1a**).
2. On a 96-well plate, distribute 6.5 µl of pre-amplification mix in each well (up to 48 wells). This plate is the 96-well single-cell sorting plate. NOTE: Using an electronic pipette is recommended for this protocol. It allows for precise and reproducible volume measurements and saves time.

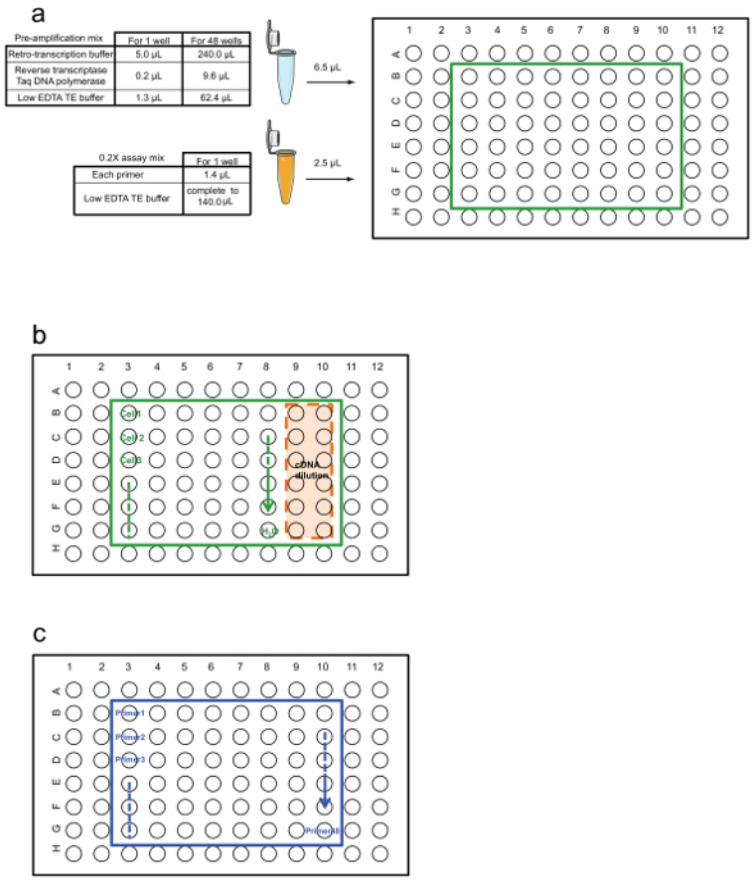

Figure 1: 96-well plate loading procedure. The loading of the 96-well plates should be done very carefully, as it will impact the rest of the experiment. (**a**) On the 96-well single-cell sorting plate, the pre-amplification mix is distributed first, followed by the 0.2x assay mix. (**b**) The record of each single-cell position should be kept on a spreadsheet. A well without a cell is called a "no input" well and can be used as a control. Two rows can be spared to control primer efficiency with cDNA dilution (sequential one-in-ten dilutions from the equivalent of 10^5 cells to one cell). (**c**) On the 96-well assay plate, the assay loading reagent is distributed first, followed by the addition of the primers. Do not forget to keep a layout of each primer position. Please click here to view a larger version of this figure.

3. Prepare a 0.2x assay mix in a 1.5 ml tube by adding 1.4 µl of each primer (primers 20x; up to 48 different probes; see **Table 1**). Adjust the final volume to 140 µl with low EDTA TE buffer.
4. Distribute 2.5 µl of the 0.2x assay mix to the 48 wells in the 96-well single-cell sorting plate containing the pre-amplification mix.
   NOTE: The final volume in each well of the 96-well sample plate should be 9 µl.
5. Seal the plate with a cover film. Vortex the plate and spin it down at 280 x g for 45 sec. Store the 96-well single-cell sorting plate at -20 °C or use it immediately.

2. Single Cell Dissociation

1. Using a CO_2 delivery system, euthanize a mouse by hypoxia. Fix the mouse on a dissection plate and open the peritoneal cavity longitudinally using scissors.
2. Recover ILCs from the liver.
   1. Prior to liver isolation, flush the liver-circulating cells.
      1. To bring out the portal vein, move the small and the large intestine to the left side of the mouse; the portal vein appears as the largest vein in the peritoneal cavity.
      2. With a 10 ml syringe and a 0.45 mm needle, flush the liver with phosphate-buffered saline medium (PBS) through the portal vein. To facilitate liver flushing, cut the gastric artery with scissors.
      3. To remove the gallbladder, pinch the base of the gallbladder with forceps and separate it from the liver with scissors.
      4. To isolate the liver, pinch the base of the liver with forceps, and using scissors, separate the liver from the rest of the peritoneal cavity.
      5. Transfer the liver in a potter tube with 5 ml of Roswell Park Memorial Institute medium (RPMI) 2% fetal calf serum (FCS).

3. Dissociate the liver with a mechanic dissociation using a pestle. Transfer the medium to a 15 ml tube. Bring the total volume to 14 ml with RPMI 2% FCS. Leave the cell suspension on ice for 15-20 min to decant the hepatocytes.
4. Recover the supernatant (without the hepatocytes) in a new 15 ml tube using a 1 ml pipette (12 ml of supernatant can be expected). Spin down at 120 x g for 7 min at 10 °C. Discard the supernatant after centrifugation.

5. Resuspend the pellet obtained in step 2.4 in 14 ml of density gradient medium (*e.g.,* Percoll 40%). Spin down at 600 x g for 20 min at 20 °C. Remove the supernatant by aspiration.
6. Resuspend the pellet in 1 ml of potassium acetate (ACK). Leave at room temperature for 1 min. After 1 min, bring the total volume to 14 ml with Hank's Balanced Salted Solution (HBSS) 2% FCS. Spin down at 120 x g for 7 min at 10 °C. Discard the supernatant.
7. Stain the cells.
    1. Resuspend the pellet in 300 µl of biotinylated antibody mix (see the **Materials Table**; add all biotinylated antibodies mentioned) and transfer the cells in to 1.5 ml tube. Leave it in the dark for 20 min at 4 °C.
    2. Bring the total volume to 1 ml with HBSS 2% FCS. Spin down at 120 x g for 7 min at 10 °C. Resuspend the pellet in 300 µl of fluorochrome-coupled antibody mix and fluorochrome-conjugated streptavidin (see the **Materials Table**; add all fluorochrome-coupled antibodies mentioned). Leave it in the dark for 20 min at 4 °C.

8. Bring the total volume to 1 ml with HBSS 2% FCS. Spin down at 120 x g for 7 min at 10 °C. Remove the supernatant using a 1 ml pipette. Resuspend the pellet in 1 ml HBSS and propidium iodide (Pi; 1:4,000).
NOTE: When using the widely expressed ILC cell-surface markers, 3 populations are defined: NKp46^{+} IL-7Rα^{-}, NKp46^{+} IL-7Rα^{+}, and NKp46^{-} IL-7Rα^{+}. All populations are sorted lineage^{-} CD3^{-} CD4^{-} CD45.2^{+}.

3. Single-cell Fluorescence-activated Cell Sorting (FACS)

1. Use FACS to sort single cells[13].
NOTE: Different cell types can be sorted on the same 96-well single-cell sorting plate (**Figure 2**).
    1. Use FACS to sort one cell per well containing the 0.2x assay mix and the pre-amplification mix on the 96-well single-cell sorting plate. Use a freshly prepared 96-well single-cell sorting plate or thaw it if stored at -20 °C.
        1. Put a sealed 96-well plate on the FACS plate holder. Use an empty 96-well plate as a test. Position the plate with the A1-well on the left and toward the experimenter.
        2. Adjust the plate holder to obtain a drop in the center of the A1-well with verification beads.
        3. Repeat step 3.1.1.2 with all the wells in line A.
        4. Remove the seal from the 96-well plate and sort 100 verification beads per well. Check for drop formation in the center of the wells.
        5. When properly adjusted, place the 96-well single-cell sorting plate on the plate holder. Draw the plate layout and sort 1 cell per well.
        NOTE: The proper positioning of each cell on the 96-well single-cell sorting plate is essential. A plate layout should be kept on a spreadsheet software. The plate layout will be used in the next several steps (**Figure 1b**)[14]. Leave one well containing 0.2x assay mix and pre-amplification mix on the 96-well sample plate without cells. This well is used as a no-input control. Leave two rows of 6 wells for a cDNA dilution. These wells are used as controls for primer efficiency (**Figure 1b**).
        6. Store the 96-well single-cell sorting plate at -20 °C or use it immediately.

4. Pre-amplification

1. Use a fresh or thawed 96-well single-cell sorting plate obtained in step 3.1.1.6. Vortex the plate and spin it down (280 x g for 45 sec).
2. Place the 96-well single-cell sorting plate on the thermocycler. Perform reverse transcription and pre-amplification as per the mentioned program in **Figure 3** and **Table 2**.

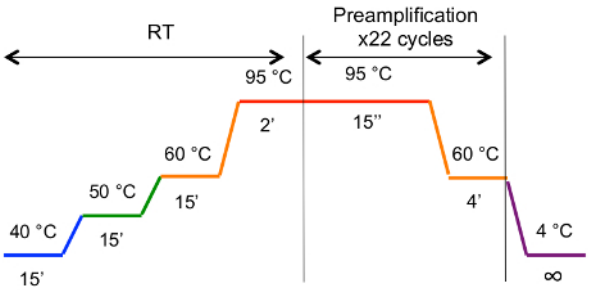

Figure 3: Pre-amplification program. In order to have enough material, pre-amplification of specific target genes on sorted single cells is required. The 96-well single-cell sorting plate is loaded on a thermocycler to follow the pre-amplification program. The pre-amplification products are then diluted with low EDTA TE buffer and can be used immediately or frozen at -20 °C. Please click here to view a larger version of this figure.

3. Dilute the pre-amplified samples by adding 36 µl of low EDTA TE buffer into each well.
4. Seal the plate with a cover film. Vortex the plate and spin it down (280 g for 45 sec). Store the pre-amplified, 96-well single-cell sorting plate at -20 °C or use it immediately.

5. Prepare a 96-well Sample Plate

1. Prepare 191 µl of master mix by adding 175 µl of qPCR master mix and 17.5 µl of sample loading reagent.

2. On a new 96-well plate, distribute 3.6 µl of master mix in each well (up to 48 wells). This is the 96-well sample plate.
3. Transfer 2.9 µl of pre-amplified cDNA from the 96-well sample plate to the new 96-well plate. Keep the same position for each sample between the 96-single cell sorting plate and the 96-well sample plate.
4. Seal the plate with a cover film. Vortex the plate and spin it down (280 x g for 45 sec). Place the new 96-well plate on ice protected from light. Store the 96-well sample plate at -20 °C or use it immediately.

6. Prepare a 96-well Assay Plate

1. On a new 96-well plate, distribute 3 µl of assay loading reagent in each well (up to 48 wells). This plate is called the 96-well assay plate.
2. Add 3 µl of primers to each well. Keep a layout on a spreadsheet software (**Figure 1c** and **Table 1**; use all primers listed in **Table 1**). The proper positioning of each primer on the 96-well assay plate is essential for the next several steps.
    1. If the number of samples is below 48, add water instead of primers to unused wells.
3. Seal the plate with a cover film. Vortex the plate and spin it down (280 x g for 45 sec). Store the 96-well assay plate at -20 °C or use it immediately.
   NOTE: To avoid repeated freezing and thawing of the primers, distribute the primers for the pre-amplification mix and for the 96-well assay plate at the same time.

7. Single-cell Gene-expression Chip

1. Place the integrated microfluidic circuit (IFC) on the bench and check the valves using the capped syringe. Open the syringe, place it perpendicular to the valve, and press firmly. The O-ring should move. Fill the chip with control line fluid (0.3 ml of tuberculin).
2. Repeat step 7.1 with the second valve.
   NOTE: No liquid should be spilled over the chip.
3. Remove the blue film from the bottom of the chip. Load the chip into the IFC controller. On the IFC controller screen, select "PRIME" and then "RUN." The control of the microfluidic lines last 10 min.
4. Eject the chip and re-seal the blue film on the bottom of the chip.

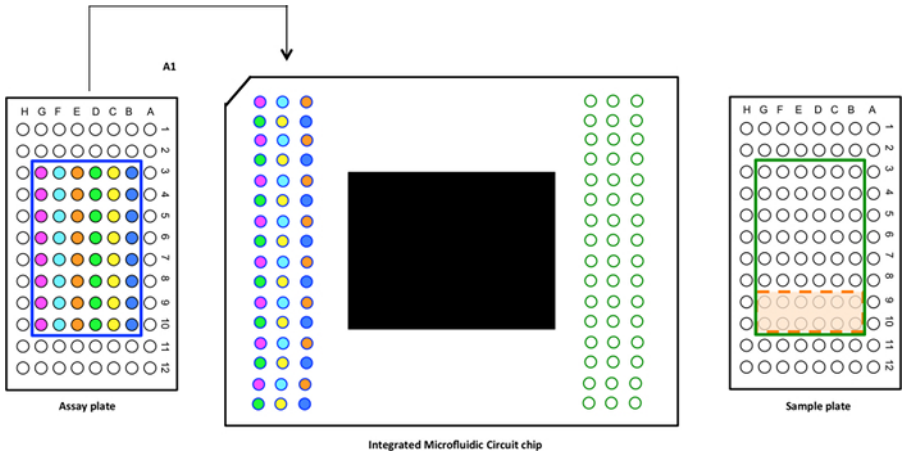

Figure 4: Single-cell multiplex gene-expression chip loading. These steps require great precision, especially during the transfer of the 96-well plate to the single-cell multiplex gene-expression chip. To avoid loading errors and misplacements, it is highly recommended to work sequentially. The volume taken for each transfer should be controlled during the pipetting process. Finally, it is important to avoid any bubbles and to remove them in case of formation. Please click here to view a larger version of this figure.

5. Using an 8-channel pipette, transfer 5 µl from the 96-well assay plate on the A1 side (left side) of the chip. Fill the left side of the chip, as indicated in **Figure 4**. Be careful to always draw the same volume into the tips.
    1. Do not create bubbles. If bubbles appear, remove them using 10 µl tips. Change tips for each well of the chip.
6. Repeat step 7.5 on the right side of the chip using 5 µl from the 96-well sample plate.
7. Remove the blue film from the bottom of the chip. Load the chip into the IFC controller. On the IFC controller screen, select "RUN SCRIPT" and then "RUN." The loading of the microfluidic lines lasts 45 min.
8. Eject the chip and re-seal the blue film on the bottom of the chip.

8. Run the Chip

1. On the microfluidic qPCR computer, select the "Data Collection" software. Once it starts, select "New Run."
2. Select "Eject," remove the blue film from the bottom of the chip, and load the chip. Place the chip to get the A1 well on the left and toward the experimenter.
3. On "Project setting," select "None" and then "Next."
4. Select "New chip run," "New chip directory" (create a folder for the experiment), and "Next."
5. On "Gene expression" select, "Passive reference = ROX" (carboxy-x-rhodamine) and "Single probe;" select the correct filter according to the primers. Select "Next."

6. Select "Browse" and select the correct program according to the primers used.
   NOTE: "Default-10min-Hotstart.pcl" is the most commonly used program for single-cell multiplex RT-qPCR.
7. Select "Start Run". The reaction takes approximately 90 min.
8. Once finished, select "Eject-Done."

9. Data Analysis

1. Open the "Real-Time PCR Analysis" software, select "File" and "Open," find the experiment folder, and select "ChipRun.bml file."
2. Click on "Analysis View," "Results Table," and "Heat Map View;" boxes marked with an "X" are below the threshold detection level and/or had bad amplification curves.
3. Name the samples. Go to "Sample Setup" and select "New SBS 96." Click on "Mapping" and select "…" Copy and paste the sample layout design from the spreadsheet software. Define the pasted layout as "Sample Name."
4. Name the assays. Repeat step 9.3 with the primer names in "Detector Setup." Define the pasted layout as "Detector Name."
5. Click on "Analysis view" and "Analyze;" the sample and primer names will be attributed automatically to the samples and primers (**Figure 7**).
6. Calculate Ct, Delta Ct, and Fold Change for each sample. Use a housekeeping gene as an internal control (here, *Gapdh*):
   $\Delta Ct = Ct_{sample} - Ct_{internal\ control}$
   $Fold\ Change = 2^{-(\Delta Ctsample-\Delta Ctnegative\ control)}$
   1. Click on "Detector Setup" and select the well with no input control (use the housekeeping gene as an internal control to normalize gene expression). Select "Editor," "define reference," and "Update."
   2. Select all wells to which the above-defined internal control will be applied. Select "Editor" and "define as Test." Select an appropriate reference gene and click "Update."
   3. Select "Sample Setup" to select the negative control well. Select "Editor" and define "Reference." Click "Update."
   4. Select "Analysis View" and "Analyze;" the Delta Ct and Fold Change will automatically be calculated.
7. Export the data. Select "File" and "Export, save as "Table Results," select the destination folder, and hit "Save" and "Exit."
8. Repeat step 9.7, but instead of "Table Results," save as "HeatMap Results."

Representative Results

Lymphoid populations display great diversity in gene expression. In this protocol, liver ILC compartment heterogeneity was investigated using single-cell multiplex RT-qPCR gene expression. Unlike other gene expression techniques, single-cell multiplex RT-qPCR gene expression allows work on several populations, even the rarest, at the same time. This specificity, coupled with a high sensitivity at the clonal level, allows for the investigation of differences in gene signatures within a population and between rare populations. As an example, the gene signatures of 3 ILC populations from the livers of 4-week-old mice were assessed. One population is frequent enough to be easily identified, whereas the two other are rare (less than 1% of lineage-negative cells) in the liver.

A 96-well single-cell sorting plate and a 96-well assay plate were prepared prior to liver dissection and dissociation (**Figure 1**). Each mix was prepared under a sterile hood and distributed with an electronic pipette for volume precision and reproducibility. All reagents were vortexed before pipetting to maintain homogeneity. After preparation, the 96-well cell sorting plate can be frozen until cell sorting, and the 96-well cell assay plate can be frozen until IFC loading.

Livers were dissected and dissociated into single-cell suspensions. Cells were stained and sorted on thawed 96-well single-cell sorting plates. Using FACS, 3 ILC populations were sorted based on widely expressed ILC markers (**Figure 2**). After cell sorting, cDNA was synthesized, and specific target genes were amplified (**Figure 3** and **Table 2**). After the pre-amplification step, cDNA was diluted in low EDTA TE buffer. Primers and cDNA from single cells were loaded onto the IFC chip (**Figures 4** and **5a**). The obtained results (**Figure 7a**) were simplified for easier reading and analysis (**Figure 7b**). Examples of properly or improperly loaded chips are shown (**Figure 5**). A FAM figure of a properly loaded chip (**Figure 6**) with different amplification signals is shown.

The results show that after proper cell sorting, pre-amplification, and loading, the ILC population appears heterogeneous for gene expression in the liver of adult wild-type mice (**Figure 7**). Using online software, cell-specific gene expression signatures (**Figures 7** and **8**) and cell population relationships (**Figure 8**) could be identified. Each sorted population has a specific gene signature enriched in gene expression. For instance, cells sorted as NKp46$^-$ IL-7Rα$^+$ have enriched expression of *Rorc*, the main transcription factor of group 3 ILCs; *Rora,* a *Rorc*-homologous transcription factor; and *Il-23r* and *Cxcr6*, two important receptors of group 3 ILC hepatic markers in the liver. The same observations can be made with NKp46$^+$ IL-7Rα$^-$ and NKp46$^+$ IL-7Rα$^+$ populations. Each cell is checked for housekeeping gene expression (*Hprt, Act*, and Gapdh) to exclude invalid wells; at least two housekeeping genes must be expressed to consider the values as valid.

Interestingly, this technique allows for the definition of two subpopulations of NKp46$^+$ IL-7Rα$^-$ based on gene signatures. Differences between those two signatures have to be validated by other sets of different functional experiments.

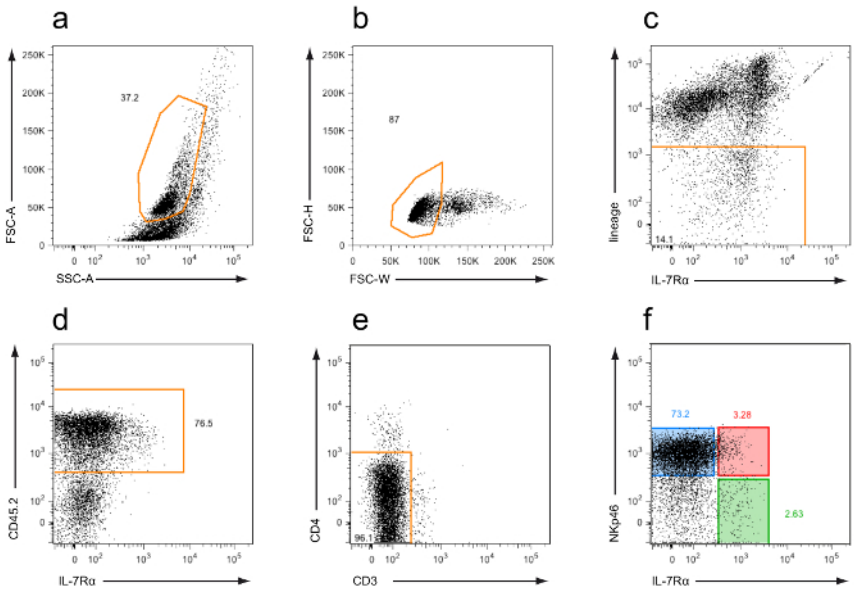

Figure 2: FACS cell strategy. Images are representative of isolated liver cells from 4-week-old mice. (**a**) FSC-A/SSC-A gating, (**b**) FSC-H/FSC-W doublet discrimination, (**c**, **d**, and **e**) alive, lineage-negative CD45.2^{+} CD4^{-} CD3^{-} cells gating. (**f**) Using widely expressed ILC cell-surface markers, we defined 3 populations: NKp46^{+} IL-7Rα^{-}, NKp46^{+} IL-7Rα^{+}, and NKp46^{-} IL-7Rα^{+}. Please click here to view a larger version of this figure.

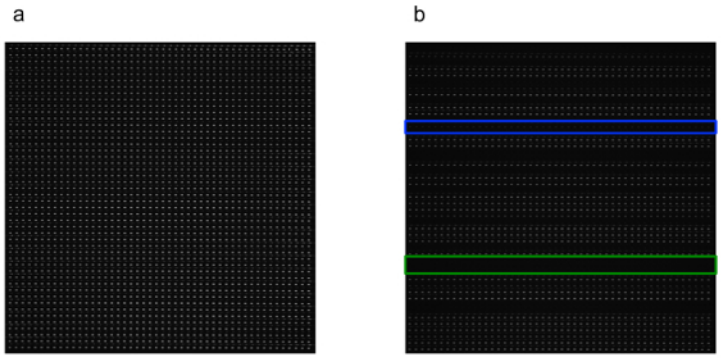

Figure 5: ROX figures of properly (**a**) and improperly (**b**) loaded chips. (**a**) Properly loaded single-cell multiplex gene-expression chip should appear with straight lines and rows. Each reaction chamber is filled and has the same dimension. (**b**) Improperly loaded single-cell multiplex gene-expression chip. Empty lines and rows of reaction chambers appear (green square), as well as bending lines (blue square). These features can be due to improper "PRIME" or "LOAD" steps, as well as to residual bubbles in the IFC wells. Please click here to view a larger version of this figure.

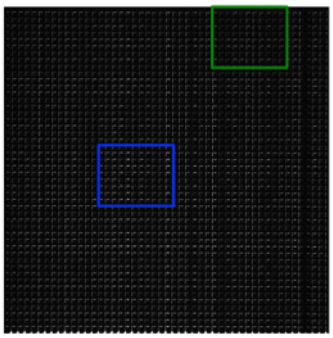

Figure 6: FAM (Fluorescein amidite) figure of a properly loaded chip. After a few cycles (depending on the samples and on the primers assessed), differences in reaction chamber brightness should appear. Reaction chambers with an amplification signal should appear brighter (blue square) than reaction chambers with no or low amplification signals (green square). Please click here to view a larger version of this figure.

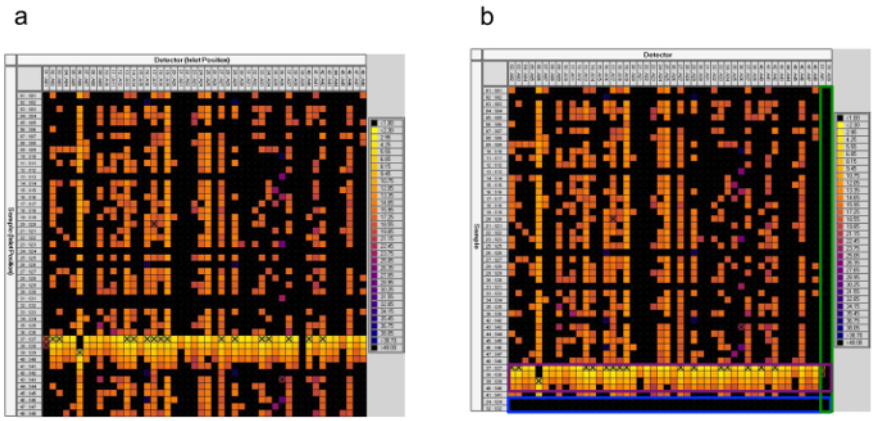

Figure 7: Heat map obtained before (**a**) and after (**b**) modifications. (**a**) Direct heat map obtained without analysis or modification of assay/ sample order. (**b**) Modified heat map obtained after sample and assay name definition. No input wells (blue square) are obtained if no amplification occurs. Inefficient primers (green square). cDNA dilution test (purple square). Please click here to view a larger version of this figure.

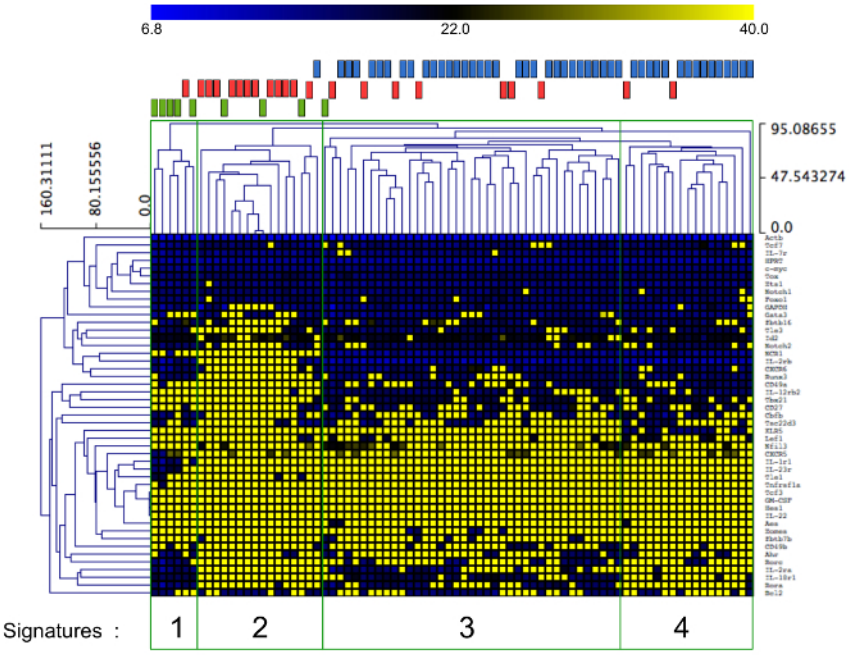

Figure 8: Single-cell population clustering. A data analysis software is used to determine the clustering between populations. Free clustering software are available online. By assessing the whole expression profile of a single cell, the software can display gene signatures and cell population relationships. Each square represents a cell: blue are NKp46^{+} IL-7Rα^{-}, red are NKp46^{+} IL-7Rα^{+}, and green are NKp46- IL-7Rα^{+}. Each population has a specific gene signature. Within the NKp46^{+} IL-7Rα^{-} population, two signatures can be seen, attesting to population heterogeneity. Please click here to view a larger version of this figure.

Primers	
Actb	*Il-23r*
Aes	*Il-2ra*
Ahr	*Il-2rb*
Bcl2	*Il-7ra*
c-myc	*Klr5*
Cbfb	*Lef1*
Cd27	*Ncr1*
Cd49a	*Nfil3*
Cd49b	*Notch1*
Cxcr5	*Notch2*
Cxcr6	*Rora*
Eomes	*Rorc*
Ets1	*Runx3*
Foxo1	*Tbx21*
Gapdh	*Tcf3*
Gata3	*Tcf7*
Gm-csf	*Tle1*
Hes1	*Tle3*
Hprt	*Tsc22d3*
Id2	*Tnfrsf11a*
Il-12rb2	*Tox*
Il-18r1	*Zbtb16*
Il-1rl1	*Zbtb7b*
Il-22	

Table 1: Primer list.

Program	Temperature	Time	Cycles	
Reverse transcription	40 °C	15 minutes	1	Reverse Transcription
	50 °C	15 minutes	1	
	60 °C	15 minutes	1	
RT enzyme inactivation	95 °C	2 minutes	1	
Denaturation	95 °C	15 secondes	22	Pre-amplification
Annealing and extension	60 °C	4 minutes	1	
Hold	4 °C	∞		

Table 2: Pre-amplification program. Each step of the pre-amplification program is shown with the complete information regarding the time, temperature, and number of cycles.

Discussion

This protocol describes how to obtain exhaustive gene expression information at the clonal level. Here, we investigated liver ILC compartment heterogeneity. After single-cell sorting of different ILC populations (based on widely expressed ILC surface markers), samples were pre-amplified for specific pre-selected genes. Then, the obtained cDNA and primers were loaded onto a multiplex RT-qPCR microfluidic chip. Finally, we obtained the expression of 48 different genes from 48 single cells. Gene expression results were analyzed via an online software to investigate gene signature and cell population relationships.

The principal advantage of this technique is the ability to assess multiple gene expression simultaneously. It also allows work at the clonal level and on very rare populations. Unlike conventional RT-qPCR methods, single-cell multiplex RT-qPCR has no averaging effect, since gene expression is assessed at the clonal level. Thus, heterogeneity within a population can be detected and analyzed. Single-cell RT-qPCR expression allows work on very rare populations, since only very few cells are needed to obtain wide information on gene expression. Moreover,

different populations of cells can be tested on the same plate. This enables the detection of differences in gene signature between populations and, with online data tools, the assessment of cell population relationships. This technique presents other advantages as well. Single-cell multiplex RT-qPCR has the benefit of the recent microfluidic advances. Volumes needed in the IFC chambers do not exceed the nanoliter-level. Thus, lower amounts of reagents are used, reducing the cost of experiments. Finally, the overall number of pipetting steps is reduced, thereby limiting the possibility of pipetting errors. The steps are simultaneous for all the cells and can be done with a multichannel pipette, enabling work in a fast and time-saving manner. Obtained results at the clonal level are reproducible, reliable, and can be used to perform robust data analyses.

Single-cell multiplex RT-qPCR does, however, present some limitations. First, this technique necessitates expensive and specific equipment, such as a microfluidic chip, a microfluidic chip controller, and a specific thermocycler. Unlike conventional RT-qPCR methods, this technique is time- and reagent-saving, since, with conventional RT-qPCR, numerous studies are needed to obtain statistically significant comparisons. In this protocol, we present a procedure to obtain gene expression for 48 genes. 96-96 multiplex RT-qPCR microfluidic chips are available. These chips can assess gene expression for 96 genes from 96 cells at the same time. During cell sorting, a minimum number of starting cells are needed, since precision and cell purity must be maximal. However, even with a small number of cells, it is still possible to sort for single-cell multiplex RT-qPCR gene expression (step 3). Finally, single-cell multiplex RT-qPCR is a sensitive method. Different tests must be performed before starting such experiments. For instance, for primer target specificity, primers should be tested for amplification efficiency and for the relative competition for the target.

Several steps of the protocol must be performed with caution. The manipulations of small volumes combined with a large number of samples and assays at the same time increase the risk of pipetting mistakes (steps 1, 4, 5, 6, and 7). All the reagents and mixes should be vortexed prior to pipetting in order to ensure a homogenous solution. Evaporation during pre-amplification (step 4) can also impact the final results. The plates should always be properly sealed with an appropriate cover film. Sorting strategy must be very precise (step 3) to avoid dead cells or multiple cells in wells within the plates. FACS sorting machines are now equipped with single-cell sorting index software that can record which specific cell was sorted in each well. This new tool will be of interest for the determination of whether gene signatures are correlated to cell-surface marker intensity. Sample and assay positions on chips must be organized meticulously, as this is essential for the experiment (steps 1, 5, and 6). Finally, the selection of primers (step 1 and 6) is a critical step. Every primer must be selected based on previously described results or expected results. A random choice of primers in the set of assessed genes could impair the final readout of the experiment. Furthermore, primers that correlate with the cell-surface marker used for cell sorting have to be used as controls with housekeeping genes.

Assessing multiplex gene expression at the clonal level offers a better characterization of the liver ILC heterogeneous compartment than in previous research. This technique, supplied by online software, describes more precisely the different subsets of ILC in the liver at homeostasis than studies using only cell-surface markers. Furthermore, it is possible to consider cell population relationships and to build differentiation networks. The gene signatures, based on single-cell gene expression, are also important tools to discern cell population features and to examine potential roles. Single-cell multiplex RT-qPCR is one of the best techniques for a gene expression assay to obtain reliable data. These data are easily manageable with bioinformatics software[15]. With respect to other techniques for gene expression studies, such as microarrays or RNA sequencing, single-cell multiplex RT-qPCR offers high sensitivity. Other approaches to single-cell analysis have been described and can be used as complementary techniques to single-cell multiplex RT-qPCR[15,16]. Furthermore, the technique can be performed with any primer combination, allowing users to look at personalized gene signatures. Many other applications can be used with this technique. For instance, time-course gene expression of cells throughout development can be done to assess the modification of the molecular profile during development. Drug effects on cells can also be investigated with drug dilution to determine the threshold of drug efficiency on gene expression.

Disclosures

The authors have nothing to disclose.

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